Basic Research Paper

Role of high mobility group box 1 in adriamycin-induced apoptosis in leukemia K562 cells

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Key words: HMGB1, leukemia, gene recombination, apoptosis

Background and Objective: High mobility group box 1 (HMGB1), a nuclear DNA-binding protein, stabilizes the structure and function of chromatin, regulates gene transcription. Recent studies found that HMGB1 is associated with the proliferation and metastasis of many tumors, including breast cancer, colon carcinoma, and melanoma, and is rich in various solid cancer tissues and immature cells. This study was to explore the role of HMGB1 in adriamycin (ADM)-induced apoptosis in leukemia K562 cells. Methods: K562 cells were transiently transfected with recombinant plasmid pcDNA3.1-HMGB1. The expression of HMGB1 in K562 cells were detected by Western blot and reverse transcription-polymerase chain reaction (RT-PCR). The 50% inhibition concentration (IC50) of ADM for K562 cells was determined by WST-8 assay. Cell apoptosis was assessed by flow cytometry. The protein level of Bcl-2 was detected by Western blot. The activities of Caspase-3 and Caspase-9 were assayed with Caspase Colorimetric Assay Kit. Results: The mRNA and protein levels of HMGB1 in K562 cells transfected with pcDNA3.1-HMGB1 were increased by about 85% and 56% respectively as compared with those in K562 cells transfected with pcDNA3.1. Overexpression of HMGB1 in K562 cells by transient transfection significantly increased the resistance to ADM; the IC50 of ADM was increased from (0.06 ± 0.00) μg/mL to (3.46 ± 0.06) μg/mL. When treated with 1 μg/mL ADM, the apoptosis rate was significantly lower in HMGB1-transfected K562 cells than in pcDNA3.1-transfected K562 cells [(12.00 ± 1.26)% vs. (44.50 ± 1.87)%], p < 0.05. Overexpression of HMGB1 in K562 cells significantly inhibited ADM-induced down-regulation of Bcl-2 protein. After treatment of ADM, the activities of Caspase-3 and Caspase-9 in HMGB1-transfected K562 cells were inhibited as compared with those in pcDNA3.1-transfected K562 cells (Caspase-3: 1.55 ± 0.06 vs. 2.55 ± 0.06 at 12 h, 1.86 ± 0.10 vs. 2.85 ± 0.06 at 24 h, p < 0.05; Caspase-9: 1.40 ± 0.08 vs. 2.03 ± 0.05 at 12 h, 1.55 ± 0.06 vs. 2.22 ± 0.05 at 24 h, p < 0.05). Conclusion: HMGB1 overexpression could inhibit ADM-induced apoptosis in leukemia K562 cells through regulating the protein level of Bcl-2 and the activities of Caspase-3 and Caspase-9.

Recent studies have found that the High mobility group box 1 (HMGB1), a nucleoprotein, is associated with tumor growth and metastasis, and is highly expressed in various solid tumors and immature cells.1 We have recently identified that serum HMGB1 level is closely related to the onset and development of leukemia in children, and HMGB1 serum level monitoring helps in determining prognosis.2 Antitumor drugs (such as doxorubicin, vincristine, arsenic trioxide) are usually the inducers of apoptosis and apoptosis is the final common pathway triggered by most chemotherapeutic drugs.3 However, it is unclear whether HMGB1 is involved in the regulation of chemotherapeutic drug-induced apoptosis of leukemia cells. In this study, we transfected HMGB1 gene into leukemia K562 cells, observed its effect on adriamycin (ADM)-induced apoptosis of K562 cells and further explored the molecular mechanisms of HMGB1 in pediatric leukemia.

Materials and Methods

Materials. K562 cells were supplied by the Cell Center in Xiangya School of Medicine of Central South University. Mycoplasma-free fetal calf serum was manufactured by Hangzhou Sijiqing Biological Engineering Material Co., Ltd. pcDNA3.1 empty vector, Trizol reagent kit and RPMI-1640 culture medium were purchased from Invitrogen Company, USA. pcDNA3.1-HMGB1 was constructed by our laboratory and no mutations were detected by sequencing analysis. Caspase-3 inhibitor Z-DEVD-FMK was purchased from Calbiochem Company, USA. The PTC-200 rev-PCR instrument was purchased from Bio-Rad Company. Avian myeloblastosis virus reverse transcriptase (AMV-RT) was supplied by TaKaRa Bio. Primers were synthesized by Shanghai Invitrogen Biological Co. For GAPDH, the upstream primer was 5'-AAG CCC ATC ACC ATC TTC CA-3' and the downstream primer was 5'-CCT GCT TCA CCA CCT TCT TG-3'; for HMGB1, the upstream primer was 5'-ATG GGC AAA GGA GAT CCTA-3' and the downstream primer was 5'-ATT CAT CAT CAT CAT CT TCT CT-3'. Rabbit anti-HMGB1 polyclonal...
antibody was supplied by BD Biosciences, mouse anti-Bcl-2 monoclonal antibody from Stressgen Biotechnologies Corporation and mouse anti-GAPDH monoclonal antibody from KangChen Bio-tech; corresponding secondary antibodies were purchased from Boster Biological Technology, Ltd.

**Methods.** Cell culture. K562 cells were inoculated in RPMI-1640 culture medium containing 10% fetal calf serum, and cultured at 37°C in an incubator containing 5% CO₂ with saturated humidity. The medium was changed every 3–4 days.

Transient transfection with HMGB1 eukaryotic gene expression plasmid. According to the operating instruction for Lipofectamine2000 liposome transfection system, cells were inoculated in 60 mm cell culture bottle after 24-hour passage. pcDNA3.1 empty vector (8 μg) and eukaryotic expression plasmid cloned with full length HMGB1 gene (pcDNA3.1-HMGB1) were added separately into 500 μL of serum-free and antibody-free RPMI-1640 culture medium, and added with 500 μL of serum-free and antibody-free RPMI-1640 culture medium containing 20 μL liposome. After incubation at room temperature for 5 min, the plasmids were well mixed with liposome, then incubated at room temperature again for 20 min. After being washed 3 times in serum-free and antibody-free RPMI-1640 culture medium, the cells were resuspended with 1 mL of the above plasmid-liposome mixture and incubated at 37°C in 5% CO₂ incubator for 4–6 h, added with 4 mL of complete RPMI-1640 culture medium and incubated every 3–4 days.

Detection of HMGB1 mRNA expression using RT-PCR. Total RNA was extracted with Trizol Reagent kit according to the manufacturer’s instructions, and reversely transcribed into cDNA by AMV-RT; 2 μL RT product was used as template. For GAPDH, the upstream primer was 5’-AAG CCC ATC ACC ATC TTC CA-3’ and the downstream primer was 5’-CCT TCA CCA CCT TCT TG-3’; for HMGB1, the upstream primer was 5’-ATG GGC AAA GGA GAT CCTA-3’ and the downstream primer was 5’-ATT CAT CAT CAT CAT CT TT CT-3’. The conditions for polymerase chain reaction (PCR) were denaturation at 95°C for 30 s, annealing at 58°C (59°C for GAPDH) for 30 s; elongation at 72°C for 30 s, 24 cycles (23 cycles for GAPDH). PCR products were analyzed with 1.0% agarose gel electrophoresis, EB stained, photographed and scanned using Band Leader software for grey scale semi-quantitative analysis.

Western blot analysis. The cells were lysed with 2×SDS lysis buffer. Protein was collected and measured by Coomassie Brilliant Bradford method. The protein lysate was separated by 100 g/L SDS-PAGE electrophoresis, transferred to membranes and blocked with 20 g/L albumin at 4°C overnight, then subsequently added with primary antibodies (rabbit anti-HMGB1 polyclonal antibody, mouse anti-Bcl-2 monoclonal antibody and mouse anti-GAPDH monoclonal antibody) and corresponding secondary antibodies, incubated at room temperature for 2 h, DAB stained, photographed, scanned using Band Leader software for grey scale semi-quantitative analysis.

Detection of 50% inhibition concentration (IC₅₀) of adriamycin using WST8 assay. The IC₅₀ of adriamycin (ADM) was detected with CCK8 cell proliferation / toxicity testing kit supplied by Dojindo Molecular Technologies according the operating instructions. K562 cells (4 x 10⁴/well) were cultured with different concentrations (0.01–100.0 μg/mL) of ADM in a 96-well culture plate (100 μL/well) for 72 h, then added with CCK-8 (10 μL/well), further incubated for 6 h. Blank wells (no cells and drug) and control wells (no ADM) were set. The absorbance (A) values at 450 nm wavelength were measured. The reference wavelength was 630 nm.

Cell survival rate = (A_{test} - A_{blank}) / (A_{control} - A_{blank}) × 100%.

**Apoptosis analysis.** The cells were collected through flow cytometry by the Beijing Dingguo Biology according to the protocol, and centrifuged at a speed of 1000 r/min at room temperature for 5 min. After the supernatant was discarded, cells were resuspended with precooled PBS buffer, centrifuged as above to remove supernatant. Cells were resuspended and fixed in 80% ethanol to achieve a single-cell suspension.

Detection of caspase activity. The activity of caspase was measured with Caspase Colorimetric Assay Kit supplied by the Nanjing Keygen Biotech according the instruction. The cells were lysed with lysis buffer (50 μL per 3 x 10⁶ cells) on ice for 20 min, centrifuged at 10,000 r/min at 4°C for 3 min. The supernatant (50 μL) was collected for protein quantification and A_{595} measurement; another 45 μL of the supernatant was mixed with 50 μL of 2 x caspase buffer and 5 μL of reaction substrate, incubated at 37°C for 4 h. A_{405} was read with spectrophotometer.

The activity of caspase = A_{405} / A_{595}.

**Statistical analysis.** The data are presented as mean ± standard deviation. t-test was used to compare the two groups, and one-way ANOVA was used for comparison among multiple groups (LSD method for comparison between any two groups). A difference was considered significant if the p value was below 0.05.

**Results.**

HMGB1 expression in HMGB1-transfected K562 cells. Compared with empty vector-transfected K562 cells, the protein
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Table 1  The effects of HMGB1 overexpression on ADM-induced apoptosis in K562 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>IC50 of ADM (μg/mL)</th>
<th>Apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected cells</td>
<td>0.06 ± 0.00</td>
<td>43.67 ± 2.58</td>
</tr>
<tr>
<td>pcDNA3.1-transfected cells</td>
<td>0.06 ± 0.01</td>
<td>44.50 ± 1.87</td>
</tr>
<tr>
<td>HMGB1-transfected cells</td>
<td>3.46 ± 0.06a</td>
<td>12.00 ± 1.26a</td>
</tr>
</tbody>
</table>

HMGB1, high mobility group box 1; ADM, adriamycin. All data are presented as mean ± SD of six experiments. *p < 0.05, vs. pcDNA3.1-transfected cells and untransfected cells.

Table 2  The effects of HMGB1 overexpression on ADM-induced upregulation of Caspase-3 and Caspase-9 in K562 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Caspase-3 (ADM, 12 h)</th>
<th>Caspase-9 (ADM, 12 h)</th>
<th>Caspase-3 (ADM, 24 h)</th>
<th>Caspase-9 (ADM, 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected cells</td>
<td>2.53 ± 0.05</td>
<td>2.88 ± 0.05</td>
<td>2.00 ± 0.08</td>
<td>2.20 ± 0.08</td>
</tr>
<tr>
<td>pcDNA3.1-transfected cells</td>
<td>2.55 ± 0.06</td>
<td>2.85 ± 0.06</td>
<td>2.03 ± 0.05</td>
<td>2.22 ± 0.05</td>
</tr>
<tr>
<td>HMGB1-transfected cells</td>
<td>1.55 ± 0.06a</td>
<td>1.86 ± 0.10a</td>
<td>1.40 ± 0.08a</td>
<td>1.55 ± 0.06a</td>
</tr>
</tbody>
</table>

All data are presented as mean ± SD of four experiments. *p < 0.05, vs. pcDNA3.1-transfected cells and untransfected cells.

The impact of ADM on survival and apoptosis of HMGB1-transfected K562 cells. The IC50 of ADM for K562 cells was significantly higher in HMGB1 group than in empty vector group and untransfected group (p < 0.05), but there was no significant difference between empty vector group and untransfected group (p > 0.05, Table 1). The apoptosis rate of K562 cells was significantly lower in HMGB1 group than in empty vector group and untransfected group (p < 0.05, Table 1).

The influence of HMGB1 on ADM-induced Bcl-2 protein expression. After the cells were treated with ADM (1 μg/mL) for 24 h, Bcl-2 protein expression in empty vector-transfected K562 cells was significantly reduced. Using caspase-3 inhibitor Z-DEVD-FMK (20 μmol/L) to pretreat the cells for 1 h, the down-regulation of Bcl-2 protein expression caused by ADM was lessened. No reduction of Bcl-2 protein expression was observed in HMGB1-transfected K562 cells (Fig. 2).

The influence of HMGB1 on ADM-induced Caspase-3 and Caspase-9 activities. After the cells were treated with ADM (1 μg/mL) for 12 and 24 h, the activities of caspase-3 and caspase-9 in K562 cells were significantly increased in empty vector group and untransfected group (p < 0.05), but significantly reduced in HMGB1 group (p < 0.05, Table 2).

Discussion

HMGB1 is a nonhistone chromosomal protein abundantly expressed in eukaryotic cells. It has both intracellular and extracellular functions. Intracellularly, HMGB1 acts as a nuclear DNA-binding protein involved in chromatin structure stabilization and gene transcription regulation, and is highly expressed in a variety of solid tumors. Extracellularly, HMGB1 has an important role in regulating multiple pathologic processes such as infection immunity, axonal growth, tumor growth and metastasis, plasminogen activation, cell differentiation, cell reconstruction and cell migration.

Leukemia is one of the most common malignancies in China. Its mortality rate ranks first in children and adults under the age of 35. Similar to other malignancies, the onset of leukemia is also a multi-factor and multi-stage process, involving abnormal expressions and functions of a variety of proteins. We had previously explored the relationship between HMGB1 and childhood leukemia, and found the results two showed that serum HMGB1 level was very low in healthy children, but significantly elevated in acute lymphoblastic leukemia patients, which returned to normal corresponding to the remission of the disease; HMGB1 protein induced the secretion of TNF-α and other cytokines in K562 leukemia cells in both time- and dose-dependent patterns. These results indicate that extracellular HMGB1 is involved in the pathologic process of childhood leukemia.

Based on our previous study, this study further explored the relationship between intracellular HMGB1 and leukemia. Our results confirmed HMGB1 expression in leukemia K562 cells, enhanced HMGB1 expression by gene transfection in K562 cells reduced the sensitivity to ADM and inhibited cell apoptosis. Therefore, intracellular HMGB1 may be an important anti-apoptotic protein in leukemia cells. Volp et al. reported that HMGB1 protein, as an anti-apoptotic protein, was highly expressed in human colon carcinomas, and the up-regulation of HMGB1 was associated with increased NF-κB activity and increased expression of anti-apoptotic protein c-IAP2.

Apoptosis is a complicated process involving two major families Bcl-2 and Caspase, both of which play important roles in apoptosis regulation. We studied the influence of HMGB1 on Bcl-2 and Caspase.

Bcl-2 family includes anti-apoptotic proteins (such as Bcl-2 and Bcl-xL) and pro-apoptotic proteins (such as Bid, Bax, and Bak). The
balance between these two determines the cell response to death-inducing signals. Anti-apoptotic protein Bcl-2 is closely associated with childhood leukemia.7-8 Bcl-2 protein level is positively correlated with the anti-apoptotic ability of cells; Bcl-2 can control cell apoptosis through regulating mitochondrial membranes and the secretion of cytochrome C.9 Bcl-2 expression is negatively regulated by P53 protein: it is reduced during apoptosis, which activates caspase-3 leading to cell apoptosis. Meanwhile, Bcl-2 protein is also one of the substrates of caspase-3; activated caspase-3 further reduces Bcl-2 protein level by degrading Bcl-2 protein. HMGB1 can regulate p53 function.5 Our study showed that HMGB1 over-expression can alleviate ADM-induced Bcl-2 expression reduction via two mechanisms. First, HMGB1 regulates Bcl-2 expression at transcription level as a synergism with p53. Second, HMGB1 inhibits caspase-3 activity, reduces the Bcl-2 protein degradation caused by activated caspase-3, and ultimately maintains a relatively high level of Bcl-2 protein and anti-apoptotic function. Brezniceanu et al.10 reported that HMGB1 could inhibit the apoptosis in yeast cells induced by Bak overexpression. Stros et al.11 reported that HMGB1 could regulate Bak gene at transcription level. Therefore, HMGB1 can regulate the expression and function of multiple Bcl-2 family proteins.

Caspase family is a class of apoptosis-specific protease, also known as cysteinyI aspartate specific protease. Caspase activation plays an essential part in apoptosis.12 Caspase activation triggers the caspase cascade reaction, then mediates cleavage of specific substrates and activation of endogenous nucleases, which causes characteristic morphologic and biochemical representation of apoptosis.12 A dozen of Caspases have been identified, among them Caspase-9 is an important initiator Caspase, and Caspase-3 is an important effector Caspase.12 Our study showed that HMGB1 over-expression can inhibit the activation of Caspase-9 and Caspase-3 induced by ADM, thereby prevent apoptosis. Volp et al.5 reported that HMGB1 overexpression in NRK1 (normal rat kidney) cells could inhibit the activation of Caspase-9 and Caspase-3 induced by Bak and UV. HMGB1 was also reported to influence the transcriptional function of apoptosis-related proteins P53 and P73.11

In summary, HMGB1 can inhibit apoptosis induced by chemotherapy drugs like ADM in leukemia cells through stabilizing Bcl-2 protein expression and suppressing Caspase-9 and Caspase-3 activities. This finding further confirmed the role of HMGB1 in leukemia and provided new clues to prevent and treat childhood leukemia.

Acknowledgements
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References